In Vitro Susceptibility of Prototheca to pH and Salt Concentration

Sara Marques · Eliane Silva · Júlio Carvalheira · Gertrude Thompson

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Abstract Prototheca sp. can assume high economic significance in the dairy industry and pose a potential risk for the public health. We investigated the in vitro susceptibility of Prototheca isolates retrieved from mastitic milk (P. zopfii and P. blaschkeae) to different pH buffers and salt concentrations using a microbroth assay adapted from the Clinical Laboratory Standards Institute guidelines. Different pH buffer solutions ranging from pH 1 to pH 12 and different sodium chloride concentrations, 4.5, 9 and 18%, were tested. P. zopfii strains presented an optimal growth between pH 5 and 9, a complete growth inhibition at pH 3, and limited growth at pH 1 and 12, whereas P. blaschkeae strains showed higher susceptibility to all pH values except for pH 3 where it demonstrated a moderate growth when compared to P. zopfii strains. When salinity was incremented, P. blaschkeae was more resistant than P. zopfii, although a reduction in growth for all strains of Prototheca was observed. This study demonstrated differences in the in vitro susceptibilities of P. zopfii and P. blaschkeae to different pH and salt concentrations and intend to be a contribution on the understanding of some of the physiologic features that can be associated with the survival of these microalgae in the environment.

Keywords Prototheca sp. · pH sensibility · Salinity sensibility

Introduction

The genus Prototheca includes unicellular achlorophyllous microalgae that belong to the family Chlorellaceae. These reproduce asexually by formation of variable numbers of sporangiospores within a sporangium [1–3]. Members of this genus are ubiquitous saprophytes and can be isolated from a variety of environmental sources such as plants, soil, drinking water, sludge, marine water, swimming pools, feces of domestic or wild animals, barn floors and meat products [4–6]. Currently five species, Prototheca zopfii, P. wickerhamii, P. stagnora, P. ulmea and P. blaschkeae have been recognized [2, 7]. From
these, *P. zopfii*, *P. wickerhamii* and *P. blaschkeae* are known to be associated with diseases in animals and humans, especially when predisposing factors occur or when the host immunologic defences are impaired [1, 2, 7, 8]. The most prevalent form of Protothecosis in animals is bovine mastitis, which generally occurs in a chronic subclinical or a mild clinical inflammatory process in the udder and affects cows that do not respond to routine therapy [9–11]. In humans, this disease presents three clinical forms, olecranon bursitis, cutaneous lesions and disseminated or systemic infections [3, 5, 12]. *Prototheca* is largely distributed globally, and cases of this infection have been reported in the five continents [11, 13, 14]. Several reports refer that these extremely resistant algae have been isolated from a great variety of conditions, from water treated with chloride to pasteurized milk [7, 9, 13, 15–17]. These studies refer only to *P. zopfii*, and no further investigations have been performed to analyze the susceptibility of the more recently described pathogenic species, *P. blaschkeae* recovered from animal sources. The objective of this study was to determine the in vitro effects of different pH buffers and salt concentrations on *Prototheca* sp. to better understand some of the physiologic characters that can be involved in its survival and propagation in the environment.

**Materials and Methods**

**Prototheca Isolates**

The field isolates of *Prototheca* used in this study belong to a major collection of several milk pathogens of the Laboratory of Infectious Diseases of Veterinary Medicine from Porto University. *Prototheca* isolates were retrieved from milk of cows with mastitis originating from different dairy herds from the northwest of Portugal, representing a prevalence of 1.31% of all isolated microorganisms. All samples were collected under sterile conditions. For diagnostic purposes, 40 μl aliquots of milk samples collected from individual quarters of the udder were streaked onto Columbia agar plates supplemented with 5% sheep blood (bioMérieux, Marcy l’Etoile, France). After 42–72 h at 37°C, plates were examined for *Prototheca* growth, and any colonies resembling this alga were subcultured once on Sabouraud dextrose agar medium (Merck Laboratories, Darmstadt, Germany). After macro and microscopical identification, *Prototheca* isolates speciation was performed by amplification of the 18S rDNA and direct sequencing of all isolates as previously described [1].

**Reagents and Solutions**

To test the pH sensitivity of the isolates, hydrochloric acid (Merck Laboratories, Darmstadt, Germany), acetic acid (Merck Laboratories, Darmstadt, Germany), sodium acetate trihydrate (Sigma, Steinheim, Germany), disodium hydrogen phosphate (Merck, Darmstadt, Germany) and sodium hydroxide (Sigma, Steinheim, Germany) were used to prepare the buffer solutions. For salinity sensibility, sodium chloride (Merck, Darmstadt, Germany) solutions were used.

**Effects of Different pH Values and Sodium Chloride Concentrations on *Prototheca* Viability**

Although there are no guidelines and interpretative criteria for *Prototheca* sp., the Clinical Laboratory Standards Institute (CLSI) M27-A2 guidelines were followed [18]. The susceptibility tests for the nine *Prototheca* isolates to different pH values and sodium chloride concentrations were adapted according to these guidelines for a microbroth assay. The experimental design included three replicates in all treatments.

Six different pH values were tested and buffer solutions were prepared according to European Pharmacopeia [19] and United States Pharmacopeia [20]. Therefore, for all buffer solutions, a concentration of 0.1 M was prepared. All solutions and buffers were prepared in the following concentrations: pH 1—hydrochloric acid buffer, 4.14 ml hydrochloric acid 37% and 495.86 ml distilled water; pH 3—acetic acid buffer, 2.86 ml acetic acid 100% and 497.14 ml distilled water; pH 5—acetate buffer, 178.5 ml acetic acid and 321.5 ml sodium acetate (0.1 M); pH 7—phosphate buffer, 378 ml disodium hydrogen phosphate and 122 ml hydrochloric acid; pH 9—phosphate buffer, 477.5 ml disodium hydrogen phosphate and 22.5 ml hydrochloric acid; pH 12—sodium hydroxide buffer, 2 g sodium hydroxide and 500 ml distilled water.

To test for salinity sensibility, three different solutions were prepared in the following concentrations: 4.5, 9 and 18% of sodium chloride corresponding
to 5-, 10- and 20-fold sodium chloride physiologic concentration, respectively.

Prototheca suspensions were prepared in sodium chloride at physiologic concentrations, with the concentration of $1 \times 10^5$ to $5 \times 10^5$ cells per ml, after which, several other dilutions were performed until reaching the working solutions ($5 \times 10^3$ to $2.5 \times 10^4$). For both tests, the following methodology was applied: 270 µl of buffer or salt solutions were introduced in the test wells in triplicate, and 30 µl of Prototheca working solution was added into the wells, with several positive and negative controls wells also included. The positive controls were Prototheca suspensions in RPMI 1640 (Gibco, Invitrogen, Paisley, UK) with 0.01% Tween 20 (Merck, Darmstadt, Germany) and the negative controls were solutions of sodium chloride, RPMI with 0.01% Tween 20, buffer solutions 5, 10 and 20 times of sodium chloride and water. Following incubation periods of 5 min, 24 and 48 h, and 1 week at 37°C in a humid chamber, 100 µl of the suspension was spread on Sabouraud dextrose agar plates and further incubated during 48 h at 37°C. Prototheca viability was determined by means of growth inhibition, counting the number of colony forming units (CFUs) observed in each plate. For the pH susceptibility testings, pH values were determined before and after the incubation periods.

Statistical Analyses

To evaluate the effects on the growth inhibition of Prototheca using the different pH buffers and the three sodium chloride solutions, the Student’s t-test was used in all comparisons with $P < 0.05$ as the threshold to determine significance of the differences.

Results

Algae Identification

The species determination of the Prototheca associated with bovine mastitis in this study and in the region was performed by molecular methods as previously described [1]. Four of the used strains were identified as P. zopfii genotype 2 and 5 as P. blaschkeae.

Effect of Different pH Buffers

Prototheca growth was variable at different pH buffers and incubation periods, as can be observed in Fig. 1. Generally, the isolates of P. zopfii presented an optimal growth between pH 5 and 9, presenting inhibition of growth at pH 3, and limited at pH 1 and pH 12. Despite, the P. blaschkeae strains presented limited and uniform growth between pH 3 and pH 12, at pH 1 their growth was completely inhibited. After 5 min of incubation both Prototheca sp. presented a similar growth at all pH values, although a significant difference ($P = 0.018$) was detected at pH 9, at which P. zopfii strains presented a higher growth than P. blaschkeae. After 24 and 48 h of incubation all P. zopfii presented a significant higher growth than P. blaschkeae strains at pH 5 ($P = 0.006$ and $P < 0.001$, respectively), 7 ($P < 0.001$ and $P < 0.001$, respectively) and 9 ($P = 0.036$ and $P = 0.043$, respectively). However, P. blaschkeae strains presented a significant higher ($P = 0.009$ and $P = 0.022$, respectively) growth than P. zopfii at pH 3. At pH 1, after 24 h, despite limited, the growth of P. zopfii was significantly higher ($P = 0.043$) than of P. blaschkeae, and the same occurred at pH 12 ($P = 0.035$) but only after 48 h of incubation. Strains of both species were further incubated during 1 week, and all strains did not grow at pH 1 and 3, and could grow at pH 5 without significant differences between them. Moreover, between pH 7 and 12 P. zopfii strains presented a significant higher ($P = 0.001$, $P = 0.007$ and $P = 0.012$, respectively) growth than P. blaschkeae. All the P. blaschkeae strains were more susceptible than P. zopfii strains to all pH values, except for pH 3. The buffer effect of all solutions was maintained during all incubation periods, being only detected small variations on the pH values (data not shown).

Effect of Different Sodium Chloride Concentrations

The results on the different salt concentrations treatments showed that the increment in salinity inhibited the growth of all Prototheca strains used in this study, with P. blaschkeae isolates presenting a slightly higher resistance than P. zopfii strains (Fig. 2) to the same treatment. All Prototheca strains showed higher growth at lower concentrations of
sodium chloride (5 and 10 times), presenting after 5 min of incubation a uniform growth at all sodium chloride concentrations. However, after 24 h of incubation, growth inhibition was detected for all strains for increased salt concentrations. Interestingly, *P. blaschkeae* was more resistant to this treatment, presenting a significantly higher (*P* = 0.001) growth at 20 times the salt concentration. After 48 h of incubation, a significant higher (*P* = 0.023, *P* < 0.001, 10 and 20 times the salt concentration, respectively) growth of *P. blaschkeae* was observed at higher sodium chloride concentrations, although this growth was moderate. After 1 week of incubation the growth of *P. zopfii* strains was completely inhibited at higher salt concentrations, but in contrast, the *P. blaschkeae* still presented some growth, although at a lower level. For all susceptibility tests in this study, all positive controls showed growth of *Prototheca* and the negative controls presented no growth (data not shown).

**Discussion**

*Prototheca* sp. are widespread worldwide throughout different environments, but are found most frequently in those with high humidity and organic matter, being its environment dissemination and perpetuation elevated [21, 22]. These ubiquitous algae are extremely resistant due to the sporopollenin included in the cell wall that allow recontamination of the environment and promote its propagation and maintenance on the environment [10, 23]. Several authors [7, 9, 13, 15–17] state that *Prototheca* have been isolated from a great variety of pH values, from water treated with chloride and from pasteurized milk, but no studies have been conducted to support these findings. Also, no characterization of the most recent specie, *P. blaschkeae*, isolated from cases of bovine mastitis, has been reported regarding their susceptibility to physical and chemical factors. Therefore, the main objective of this work was to evaluate the in vitro growth of *P. zopfii*, *P. blaschkeae* and their susceptibility to chloride concentration. This study also focused on the evaluation of *Prototheca* growth submitted to different pH values during different incubation periods.
susceptibility of *P. zopfii* and *P. blaschkeae* isolates recovered from bovine mastitis to the effects of different pH buffers and salinity concentrations.

Results of our study determined that *Prototheca* sp. presented variable sensibility to the pH buffers tested, which may suggest different environmental survival capability between species, nevertheless the well-known biologic differences. *P. zopfii* presented a significantly higher (*P* < 0.04) resistance between pH 5 and 9 than *P. blaschkeae*, demonstrating that it was not only able to survive under these pH values, but also presented a high multiplicity rate under those conditions, even after 1 week of incubation when a slower growth could be expected due to lack of nutrients. Moreover, at pH 12, *P. zopfii* was able to grow, however, at a lower rate when compared to lower pH values and could maintain cell division at least for 1 week. At pH values lower than 5, *P. zopfii* showed a lower multiplication rate, presenting at pH 1 the capacity to survive for 48 h, but was completely inactivated over this period of time. At pH 3, *P. zopfii* growth was completely inhibited after 24 h of incubation. On the other hand, *P. blaschkeae* could grow between pH 3 and 12 for at least 48 h, presenting the optimum growth conditions between pH 5 and 7. Around pH 3 and between pH 9 and 12, *P. blaschkeae* could grow demonstrating moderate resistance. Although its growth rate was near zero, the reasons for this could be that the strains could not rapidly multiply or may have entered in a quiescence condition. At pH 1, *P. blaschkeae* growth was inhibited after 24 h, and it was able to survive between pH 3 and 12 for 1 week, although its multiplication capacity was absent for all pH conditions except to pH 5 where a higher multiplication rate could be observed. The results of this study show that *P. zopfii* presented a higher multiplicity capacity in all pH buffers except to acetic acid. These data suggest that both species, but especially *P. zopfii*, are able to multiply under very adverse conditions such as pH 12.

The increment of salinity concentration in solutions was directly proportional to growth inhibition, however, with a major impact on *P. zopfii* than on *P. blaschkeae* which can lead to speculate that the sporopollenin content of the cell wall in *P. blaschkeae*...
is higher than that of *P. zopfii*. Although the growth inhibition of *P. zopfii* was detected only after 1 week of incubation at 9% of sodium chloride, its total growth inhibition was observed after 24 h of incubation at a higher concentration (18%). Therefore, *P. zopfii* can only multiple at lower sodium chloride concentrations, 4.5%. On the other hand, *P. blaschkeae* presented only a reduction on the multiplication rate which can be speculated that this alga can be found in environments with high salt concentrations when compared to *P. zopfii*.

In conclusion, *P. zopfii* can survive and propagate in environments with pH values between 5 and 12, and also at 4.5% of sodium chloride concentrations. On the other hand, *P. blaschkeae* could survive and multiply at high salinity concentrations, up to 18% of sodium chloride, but showed more susceptibility to pH buffers, multiplying at pH 5 and at a lower capacity at pH 9 and 12.

This is the first study that compares the susceptibility of *P. zopfii* and *P. blaschkeae* strains to different pH values and sodium chloride concentrations. Due to the limited number of strains used in the study (which are representative of the sub-regions of the Norwest region), the results herein reported imply the need for further investigations using isolates from different regions. The generated knowledge is a contribution on the understanding of some physiologic characters of these algae that may explain its capacity to survive and perpetuate in different environment conditions.

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